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Innate Immune Proteins and Early Innate Immune Response of Channel Catfish (Ictalurus Punctatus)

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INNATE IMMUNE PROTEINS AND EARLY INNATE IMMUNE RESPONSE OF CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

by

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Abstract

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The channel catfish (*Ictalurus punctatus*) is susceptible to bacterial and viral infections acquired from its pond environment. The innate immune proteins mannose-binding lectin (MBL) and lysozyme were studied in two different groups of channel catfish aged 2, 4, 6, 9, and 12 months-old. The two groups were maintained at a mean temperature of 27 ^{0}C and were one-year apart in their bleedings. Dot-blot enzyme linked immunosorbent assay for MBL and turbidometry lysozyme quantitative assays were done to determine the two innate immune proteins. The greatest increases in mean MBL and mean lysozyme concentrations were seen at 4 months. Two month-old catfish were comparable with 12 month-old catfish in their concentrations of MBL and lysozyme ($p < 0.05$). A decrease was seen at 6 and 9 months for MBL and at 9 and 12 months for lysozyme. Mean protein of 26.7 mg/ml and mean albumin/globulin ratio of 0.7 were determined for 4, 6, 9, and 12 month-old catfish. This study provided a foundation for understanding these innate immune proteins and the early immune response of channel catfish.

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Introduction

Immunity is a protective response needed for defense against harmful bacteria, viruses, and fungi. Immunity is mediated by two systems called innate immunity and adaptive immunity. The evolutionary type of immunity is innate immunity and offers the main resistance to pathogens within the first few hours and days (Fujita, 2002). In order to increase the likelihood for survival and elimination of invasive microbes, enhancement of innate defenses such as opsonization, cell lysis, and phagocytosis is needed (Demurs & Bayne, 1997). This natural immunity or innate immunity recognizes microbes and provides protection until an adaptive immune response can be mounted (Holland & Lambris, 2002). The innate immune system is the only defense mechanism in invertebrates and plays an instructive role in higher vertebrates (Magnadottir, 2006). The teleost (bony) fish bridge the innate and adaptive immune responses and are the first class of vertebrates with both of these systems (Whyte, 2007). Though both immune systems are present, innate immunity occupies a more important role for initial protection against pathogens (Ullal, Litaker, & Noga, 2008) and has been used as a model for studying the immune system in lower vertebrates (Moore & Hawke, 2004). Studies of genes that are innate-immune related like pattern recognition receptors, antimicrobial peptides, lectins and complement proteins have been studied in the channel catfish (*Ictalurus punctatus*). This information helps for a better understanding of the innate immune system (Gao et al., 2012). The channel catfish is an important aquaculture species and is subjected to intensive pond-rearing conditions thus increasing their risk of exposure to many pathogens (Bilodeau, Small, Wise, & Wolters, 2005).

Only limited studies have been done on innate defensive responses at different ages in teleost fishes (Maule, Schreck, & Kaatari, 1987). The majority of multicellular organisms in existence (approximately 98.6%) have only innate immunity, indicating that an adaptive immune response is not necessarily needed for survival (Watts, Munday, & Burke, 2001). An innate immune response is therefore an effective immune response for survival of animals which do not have a well-developed adaptive immune response.

Innate immunity is the first line of defense of an animal. The innate defenses include barriers such as skin, mucus membrane surfaces and proteins with antimicrobial properties; such as, mannose-binding lectin (MBL) and lysozyme together with phagocytic cells that specialize in phagocytosis. This type of innate immunity is non-specific or non-adaptive as it does not change irrespective of the number of times the animal has encountered an infection (Pinchuk, 2002). The two main innate immune proteins of our research interest were MBL and lysozyme.

The soluble plasma lectins are a first line of host defense. Collectins are soluble lectins found in mammals, birds, and fish (Vitved, Holmskov, Koch, & Teiner, 2000). The collectins have carbohydrate recognition domains that recognize specific carbohydrate substrates on the surfaces of bacteria, viruses and fungi. These specific substrates are absent on higher eukaryote cells (Fujita, 2002; Gadjeva, Takahashi, & Thiel 2004; Jack & Turner, 2003; Turner, 2003). Mannose-binding lectin is a C-type lectin and is an important component of innate immunity in mammals. Mannose-binding lectin increases in response to an infection or inflammatory response. It acts as an opsonin for phagocytosis and also activates the complement-mediated lectin pathway of

the innate immune response (Hoffmann, Kafatos, Janeway, & Ezekowitz, 1999; Suckale, Sim, & Dodds, 2005). In serum, MBL activates the lectin pathway of the complement system by binding to the mannose-binding lectin-associated serine proteases (MASPs) (Russell & Lumsden, 2005; Presanis, Kojima, & Sim, 2003; Tsutsumi, Takahashi, & Sumida, 2005; Turner, 2003). Mannose-binding lectin is complexed with two serine proteases called MBL-associated serine proteases (MASP-1 and MASP-2). Activation of MASPs lead to cleavage of C4 and C2 into C4bC2a or C3 convertase. This results in the killing of microorganisms by activating the membrane attack complex C5b-C9 (Takahashi, Takayama, Hatsuse, & Kawakami, 1993; Sato, Endo, Matsushita, & Fujita, 1994; Thiel, Vorup-Jensen, & Strover, 1997). This activation kills Gram-negative bacteria and enveloped virus (Owen, Punt, Stranford, & Jones, 2013). Mannose-binding lectin is a recognition unit leading to antibacterial activity and opsonin-enhanced macrophage activity (Ewart, Johnson, & Ross, 1995; Ottinger, Johnson, Ewart, Brown, & Ross, 1999). Mannose-binding lectin could thus play an important role in protection to many pathogens of juvenile channel catfish, like *Edwardsiella ictaluri* and channel catfish herpesvirus.

Lysozyme is another important innate immune protein investigated here. Lysozyme is an enzyme found in serum and mucosal secretions of animals that has an antimicrobial effect. In mammals and jawed fish, lysozyme lyses the cell walls of Gram-positive bacteria. In bony fish, lysozyme is a major component in resistance to bacterial infections including Gram-positive and Gram-negative bacteria (Grinde, Lee, Poppe, & Salte, 1988; Watts, Munday, & Burke, 2001).

The channel catfish is used in aquaculture in the Southeast U.S. and is susceptible to bacterial and viral infections acquired from its pond environment. Channel catfish less than one year old are the most susceptible to infections. These bony fish belonging to the infraclass Teleostei have a characteristic forked caudal fin and grow to a size of 15-24 inches. The channel catfish are freshwater fish that grow best in warm water. They spawn during the month of May and continue to spawn as late as August, and male catfish guard the nests. They are bottom feeders in the natural environment and mainly feed on aquatic insect larvae, crayfish, mollusks and small fish (Alabama Department of Conservation and Natural Resources; SRAC, 1988).

Edwardsiella ictaluri is a Gram-negative bacterium that causes enteric septicemia of catfish (ESC) (Hawke, 1979). Enteric septicemia of catfish is the most prevalent disease affecting farm-raised channel catfish in the United States and is responsible for 50% of the total losses to catfish farmers each year (USDA, 1997). Enteric septicemia of catfish in affected catfish causes red and white ulcers on the skin and lesions in the cranial foramen (Hawke, Durborow, Thune, & Camus, 1998). This is a host specific disease with outbreaks usually occurring in the water temperature range of 22-28 degrees Celsius. The channel catfish most affected are fingerlings entering their second growing season. The susceptibility to ESC is more prominent in younger juvenile catfish than in older catfish (Jesse, 2008).

In young channel catfish, acute to chronic hemorrhagic disease is caused by channel catfish virus (Wolf & Darlington, 1971). Channel catfish virus (CCV) is a member of the Alphaherpesvirinae subfamily and is an Ictalurid Herpesvirus type 1 virus (Kucuktas, Brady, & Tuzun, 1998). Channel catfish virus was first isolated by Fijan

(1968). Channel catfish are a natural host to CCV with fry and fingerlings being highly susceptible (Plumb, Schachte, Gaines, Peltier, & Caroll, 1974). The infection occurs through gills and the intestine (Hedrick, Groff, & Mcdowell, 1987; Camus, 2004). Water temperature can play an important role in infection and mortality. A temperature of 28^0 C or greater results in infection. A decrease in this temperature decreases the mortality rate (Plumb, 1973). Symptoms of CCV include swollen abdomen, bulging eyes, and distention of the gill areas (Camus, 2004).

Channel catfish are an important aquaculture species and a good source of protein food. Poor water quality and environmental stress factors make catfish susceptible to many pathogens. Juvenile channel catfish are more highly susceptible to pathogens when compared with older catfish. This could possibly be due to an inadequate innate immune response and a maturation lag in the adaptive immune response.

The innate immune response could play an important role against initial pond infections in juvenile channel catfish. Determination of innate immune protein levels in channel catfish less than 1-year old can help to better understand their early susceptibility to infections. Channel catfish have a high susceptibility to infections in the late Spring and Summer. Seasonal variations can influence the innate immune protein levels in channel catfish raised in a pond environment (Ourth & Rose, 2011).

The innate immune response in channel catfish less than 1-year old has not been well studied. Two major innate immune proteins are mannose-binding lectin and lysozyme. The following research aims provided to a better understanding of the maturation of these innate immune proteins in juvenile channel catfish:

(1) Determine levels of mannose-binding lectin in two groups of channel catfish aged 2, 4, 6, 9, and 12 months-old that were one-year apart in their bleedings.

(2) Determine levels of lysozyme in two groups of channel catfish aged 2, 4, 6, 9, and 12 months-old that were one-year apart in their bleedings.

(3) Determine total protein concentrations, albumin and globulin concentrations and albumin/globulin ratios in two groups of channel catfish aged 2, 4, 6, 9, and 12 months-old that were one-year apart in their bleedings.

Channel catfish are susceptible to bacterial and viral infections acquired from their pond environments. Two groups of channel catfish aged 2, 4, 6, 9, and 12 months-old that were 1-year apart in their bleedings were studied for their levels of the above two innate immune proteins. This study will provide a better understanding of these innate immune proteins and the early innate immune response in channel catfish.

Materials and Methods

Catfish Serum

Serum pools from channel catfish (Mississippi Select Strain) were obtained from the Warmwater Aquaculture Research Unit, USDA, ARS, Stoneville, MS. The catfish were maintained in indoor tanks at an average temperature of 26.8 ^{O}C. Two major innate immune proteins were studied in sera of two different groups of channel catfish aged 2, 4, 6, 9, and 12 months-old that were one-year apart in their bleedings. Each monthly bleeding was a serum pool of 10 catfish.

Dot-Immunoblot Enzyme-Linked Immunosorbent Assay for Catfish Serum Mannose-Binding Lectin

A Dot-Immunoblot Enzyme Linked Immunosorbent Assay (ELISA) was done using a dot-blot microfiltration apparatus to determine serum concentration levels of MBL in the two groups of channel catfish aged 2, 4, 6, 9, and 12 months-old that were one-year apart in their bleedings .The primary antibody used was guinea pig antirabbit-MBL IgG. The dot-blot apparatus (Bio-Rad, Richmond, CA) was used according to their immunoassay procedure and previously using this technique (Ourth, Nara, & Chung, 2005; Ourth, Nara, & Simco, 2007; Ourth, Rose, & Siefkes, 2008). Fifty μl of a 1:8 dilution of catfish serum were spotted on nitrocellulose membrane in the dot-blot apparatus and vacuum applied. The nitrocellulose membrane was removed from the apparatus and transferred to a container. Non-specific protein sites were blocked with 1% purified casein solution in Tris buffered saline, pH 7.5 (TBS) for 60 min. After washing the membrane three times with TBS, 10 ml of a 1:200 dilution in TBS of the guinea pig antirabbit-MBL IgG was added and incubated for 60 min. at room temp. as the primary antibody. After washing the membrane again three times in TBS, a 1:500 dilution in TBS of rabbit antiguinea pig IgG-horseradish peroxidase conjugate (Sigma, St. Louis, MO) was added and incubated for 60 min. at room temp. as the secondary antibody. This was followed by washing the membrane three times in TBS. A 3,3'-diaminobenzidine solution was used to develop the brown colored product for 10 min. and the reaction stopped with water. Image J scanning for color density of the dot-blots was done. Mannose-binding lectin concentrations (μg/ml) were obtained using a standard curve of mannose-binding lectin concentrations (μg/ml) vs. background corrected inverse

density units as determined by the ELISA technique (Ourth & Rose, 2011).

Lysozyme Level Determination by Turbidometry Assay

Lysozyme levels were determined in channel catfish sera of two different groups of catfish that were one year apart in their bleedings. Hen egg white lysozyme (Sigma, St. Louis, MO) standards (100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml, 3.125 μg/ml, 1.56 μg/ml, 0.78 μg/ml and 0.39 μg/ml) were prepared in 0.1 M sodium phosphate buffer, pH 7.2. A suspension of *Micrococcus luteus* (0.20 mg/ml) was also prepared in 0.1 M sodium phosphate buffer, pH 7.2. Eighty-seven μl of the *M. luteus* suspension and 3 μl of catfish serum were added to microcuvettes. Changes in turbidity were measured and the decrease in absorbance at 450 nm was recorded at 15s intervals for 5 min. Lysozyme quantitative levels were determined by turbidometry assay (Demurs & Bayne, 1997). The highest decrease in absorbance at 450 nm and the concentrations were quantified from a slope obtained from a standard curve.

Serum Protein Determination by Bicinchoninic Acid Assay

Total protein concentrations were determined using the Bicinchoninic Acid Assay (BCA) protein assay (Pierce Chemical Co., Rockford, IL). A 50:1 working reagent solution was mixed with 5 μl of channel catfish serum and with the 5 protein standards. Bovine serum albumin was used as the protein standard. Absorbance was measured at 562 nm. A standard curve was prepared using the average of the blank-corrected absorbencies for each BCA standard verses each standard concentration in μg/ml. Absorbencies for each serum sample were determined. Protein concentration in μg/ml was determined from the slope of a standard curve.

Albumin Concentration Determination by Albumin Assay

Albumin concentrations were determined using the Pointe Scientific assay (Fisher Scientific, Pittsburg, PA). Five μl of channel catfish serum were transferred to a tube containing 1 ml of Bromcresol Green reagent. Tubes were incubated for 1 min. at room temp. and absorbances read at 630 nm. Albumin concentrations (mg/ml) were calculated using a formula equation.

Globulin Concentration Determination

Globulin concentrations were obtained by subtracting the albumin concentrations from the total protein concentrations.

Albumin/Globulin Ratios

Albumin/Globulin (A/G) ratios were determined by dividing albumin concentrations by the globulin concentrations.

Statistical Analysis

Bonferroni's multiple comparison statistical test one-way Anova analysis was done by Prism software in the two groups of channel catfish aged 2, 4, 6, 9, and 12 months-old. The data for MBL and lysozyme were determined for statistical significance ($p < 0.05$).

Results

Background Corrected Inverse Density and Mannose-Binding Lectin Concentrations

Background corrected inverse density (BCID) units and mannose-binding lectin (MBL) (μg/ml) concentration levels were determined in channel catfish aged 2, 4, 6, 9, and 12 months-old in Groups 1 and 2 that were one year apart in their bleedings.

Background corrected inverse density units were determined with Image-J analysis by measuring the density dots obtained by ELISA technique (Table 1; Figures 1 and 2) (Ourth, Nara, & Simco, 2007). Quantitative analysis of MBL concentrations were obtained from a standard curve of background corrected inverse density units vs. MBL concentrations (μg/ml) (Table 1; Figures 3 and 4) (Ourth & Rose, 2011). Mean BCID units and mean MBL (μg/ml) concentrations were calculated by using the data from Table 1 to construct Table 2 and Figures 2 and 4. The greatest increase in MBL and BCID units was seen at 4 months of age in both Groups 1 and 2 (Figures 1, 2, 3, and 4). A decrease in MBL and BCID units was seen at 6 months and 9 months when compared with all the other age groups (Figures 1, 2, 3, and 4). Both the 2 month and 12 month old catfish were very similar in their BCID units and MBL concentrations (μg/ml) (Figures 1, 2, 3, and 4). An increase in mean BCID units (Figure 2) and in mean MBL concentrations (μg/ml) (Figure 4) was seen at 4 months of age. Mean BCID density units (Figure 2) and mean MBL concentrations (μg/ml) (Figure 4) were similar in 6 month (10.6 μg/ml) and 9 month-old (10.6 μg/ml) catfish. There were no statistical significant differences ($p < 0.05$) for MBL levels between 2 month and 12 month-old and 4 month and 12 month-old channel catfish. The data indicate that channel catfish are competent in producing mean concentrations of the innate immune protein MBL at 2 months (21 μg/ml) of age that were comparable with the concentrations of MBL found for 12 month-old (19.9 μg/ml) catfish.

Lysozyme

Lysozyme concentrations (μg/ml) of Groups 1 and 2 of channel catfish aged 2, 4, 6, 9, and 12 months-old that were one-year apart in their bleedings were determined. Concentrations were measured by lysozyme turbidometry assay (Demurs & Bayne, 1997). A standard curve plotted between lysozyme standard concentrations (μg/ml) vs. absorbance at 450 nm was done. Absorbances of the hen egg white lysozyme standards (Sigma, St. Louis, MO) were calculated by measuring absorbance in the UV-visible at 450 nm. The R^2 value of the standard graph obtained was 0.997 and the slope was y = 2E-05x + 0.0001 (Table 3; Figure 5). Absorbances for different ages was obtained by turbidometry assay and the lysozyme concentrations for the different ages of catfish were calculated from the slope of the standard curve (Table 4; Figure 6). Mean lysozyme concentrations (μg/ml) were calculated for both Groups 1 and 2 (Table 5; Figure7). Group 1 showed higher variations in lysozyme concentrations when compared with Group 2 (Tables 4 and 5; Figures 6 and 7). The highest rise (4-times) in lysozyme was seen in Group 1 at 4 months (20 μg/ml) of age (Figures 6 and 7). A rise in lysozyme concentration was seen at 6 months of age in both Groups 1 and 2 (Figures 6 and 7). Two-month (5 μg/ml) and 4-month (5 μg/ml) lysozyme concentration levels in Group 2 were the same as found at 9 months and 12 months of age in both Groups 1 and 2 (Figures 6 and 7). There were no statistical significant differences ($p < 0.05$) for lysozyme levels among all the ages of channel catfish. The data indicate that channel catfish are competent in producing mean concentrations of the innate immune protein lysozyme at 2 months (7.5 μg/ml) of age that are comparable with the lysozyme

concentrations found for 12 month-old (5 μg/ml) catfish.

Total Serum Protein Concentrations (mg/ml), Albumin Concentrations (mg/ml), Globulin Concentrations (mg/ml), Albumin/Globulin Ratios

Total protein concentrations (mg/ml) , albumin concentrations (mg/ml), globulin concentrations (mg/ml) and albumin/globulin ratios of channel catfish aged 2, 4, 6, 9, and 12 months-old of Groups 1 and 2 that were one-year apart in their bleedings were determined. Total protein concentrations (mg/ml) were determined for both Groups 1 and 2 by the BCA assay (Pierce Chemical Co., Rockford, IL) (Table 6). Albumin concentrations (mg/ml) were determined based on the protocol of Pointe Scientific assay (Fisher) (Table 6). Globulin concentrations (mg/ml) were calculated by subtracting albumin concentrations from the total protein concentrations (Table 6). Albumin/Globulin ratios were determined by dividing the albumin concentrations with the globulin concentrations (Table 6). The two month-old catfish values for both Groups 1 and 2 were not determined due to insufficient serum. Mean total protein concentrations, mean albumin concentrations, mean globulin concentrations and mean albumin/globulin ratios were determined using both Groups 1 and 2 (Table 7). The highest total protein concentration (34.8 mg/ml) was seen at 12 months of age. A mean of 0.7 for the albumin/globulin ratios was found for the 4, 6, 9, and 12 month-old channel catfish. This protein data indicated the overall health of the channel catfish.

Discussion

Mannose-binding lectin and lysozyme were the two innate immune proteins studied. Results indicate that the innate immune protein response is well developed in

juvenile channel catfish less than 6 months old and could be important in their protection against infections.

A comparative study of innate immune protein levels was done in juvenile channel catfish aged 2-6 months-old and in channel catfish aged 6-12 months-old. This study provided a comparison then between juvenile and 12 month-old channel catfish to clarify the early innate immune response of channel catfish. Avtalion, Wojdani, Malik, Shahrabani, and Duczyminer (1973) found that 3 month-old juvenile carp immunized with BSA or O-antigen *Salmonella* had no antibody response. This finding emphasizes the significance of the innate immune proteins investigated here with regard to the 2 month and 4 month-old channel catfish. These findings indicate the importance of the innate immune response in juvenile channel catfish.

The greatest increase in BCID units and MBL levels was seen in 4 month-old channel catfish in both Groups 1 and 2 (Table 1; Figures 1, 2, 3, and 4). A decrease in BCID units and MBL levels was seen in 6 and 9 month-old channel catfish in both Groups 1 and 2 (Table 1; Figures 1, 2, 3, and 4). The BCID units and MBL levels with 2 and 12 month-old channel catfish were very similar (Table 1; Figures 1, 2, 3, and 4). The mean BCID units and mean MBL levels increased at 4 months (26.9 μg/ml) of age with a drop seen at 6 month (10.6 μg/ml) and 9 months (10.6 μg/ml) of age (Table 2; Figures 2 and 4). Levels of MBL and BCID units increased at 12 months (19.9 μg/ml) and nearly equaled the concentration found in 2 month-old (21 μg/ml) channel catfish (Table 2; Figs. 2 and 4). Background corrected inverse density units were measured by Image-J

analysis using a previously described procedure (Ourth, Nara, & Simco, 2007). Mannose-binding lectin levels were quantified in μg/ml by using a standard curve (Ourth & Rose, 2011). There were no statistical significant differences ($p < 0.05$) for MBL levels between 2 month and 12 month-old and 4 month and 12 month-old channel catfish.

Lysozyme levels were quantified using a standard curve (Table 3; Figure 5). Higher variations in lysozyme levels were observed in Group 1 when compared with Group 2. The highest increase (4-times) in lysozyme levels was seen in 4 month-old (20 μg/ml) channel catfish in Group 1 (Table 4; Figures 6 and 7). Lysozyme levels decreased in 6 month (15 μg/ml) and 9 month-old (5 μg/ml) channel catfish in Group 1 (Table 4; Figures 6 and 7). Lysozyme levels were similar with 9 and 12 months-old channel catfish in Group 1 (Table 4; Figures 6 and 7). In Group 2, the highest increase was seen in 6 month-old channel catfish, but the increase was not as great as that seen with the 4 month-old channel catfish in Group 1 (Table 4; Figures 6 and 7). Lysozyme levels in 2 and 4 month-old channel catfish were similar to those of 9 and 12 month-old channel catfish in Group 2 (Table 4; Figures 6 and 7). Mean lysozyme levels increased at 4 months and were the same as found with 6 month-old channel catfish (Table 5; Figure 7). Mean lysozyme levels decreased at 9 months and were similar to the level of 12 month-old channel catfish (Table 5; Figure 7). Mean lysozyme levels of 2 month-old (7.5 μg/ml) channel catfish were similar to the lysozyme levels of 12 month-old (5 μg/ml) channel catfish. There were no statistical significant differences ($p < 0.05$) for lysozyme levels among all the ages of channel catfish. Bilodeau, Small, Wise, and Wolters (2005) found peak plasma lysozyme activity in resistant and susceptible adult channel catfish (USDA

103 strain) at 5 days following exposure to *Edwardsiella ictaluri*.

Total protein concentrations, albumin concentrations, globulin concentrations and A/G ratios were studied in channel catfish and indicated the overall good health status of the channel catfish (Tables 6 and 7) (Hrubec & Smith, 1999; Ellsaesser & Clem, 1987). Serum protein concentration at 4 months of age was 27.4 mg/ml. The highest total protein concentration (34.8 mg/ml) was seen in 12 month-old channel catfish. Mean protein of 26.7 mg/ml and mean albumin/globulin ratio of 0.7 were determined for 4, 6, 9, and 12 month-old catfish (Table 7).

A supplementary study was done to check the consistency of the levels of MBL. We therefore determined the levels of MBL in channel catfish aged 5, 6, and 7 months-old in a separate study (Table 8). A similar pattern of MBL levels was found when compared with levels found for catfish aged 2, 4, 6, 9, and 12 months-old (Tables 1 and 2; Figures 3 and 4). Mannose-binding lectin concentration increased at 5 months (Table 8) which was found to be consistent with the mean MBL concentration for 4 month-old channel catfish (Table 2; Figure 4). Mannose-binding lectin level decreased at 6 months (Table 8) and was consistent with the mean MBL level found for 6 month-old channel catfish (Table 2; Fig. 4). Mannose-binding lectin level slightly increased at 7 months (Table 8) and was consistent with the 9 month-old channel catfish (Table 2; Figure 4). This data (Table 8) provided consistency to the pattern of MBL levels found for the channel catfish aged 2, 4, 6, 9, and 12 months-old (Tables 1 and 2; Figures 1, 2, 3, and 4).

The channel catfish were maintained in indoor tanks. A temperature range of 26.3 ^{O}C – 27.3 ^{O}C was observed (Table 9). The mean temperature over a one-year period was 26.8 ^{O}C (Table 9). A decrease of 56 % in μg/ml of channel catfish MBL was found

between the November-February months and July-September months (Table 9). Lysozyme levels between the November-February months and July-September months were nearly the same (Table 9). The lowest levels of MBL (10.6 μg/ml) were seen in the months of November and February, and the highest level of MBL (26.9 μg/ml) was seen in September (Table 9). Enteric septicemia of catfish in pond-raised catfish occurs primarily in the Spring and early Fall in the Southeast USA. A decrease of 33% in μg/ml MBL of adult channel catfish was seen between the late Fall and Spring seasons which could influence their susceptibility to infection (Ourth & Rose, 2011).

This study determined levels of the innate immune proteins, MBL and lysozyme, in channel catfish aged 2, 4, 6, 9, and 12 months-old. It was found that the levels of these innate proteins in juvenile channel catfish at 2 months of age were comparable to those levels found with 12 month-old adult channel catfish. These studies provide further evidence that juvenile channel catfish are innately immunocompetent in having an early innate immune response for protection against infections.

Conclusion

The greatest increase in MBL was seen at 4 months of age for channel catfish in both Groups 1 and 2. A decrease in MBL was seen at 6 months and 9 months of age when compared with all the other age groups. Both 2 month and 12 month old channel catfish were very similar in their BCID units and MBL concentrations (μg/ml). Group 1 showed higher variations in lysozyme concentrations when compared with Group 2 channel catfish. The highest rise (4-times) in lysozyme was seen in Group 1 channel catfish at 4 months of age. A rise in lysozyme concentration was seen at 6 months of age

in both Groups 1 and 2. Two-month and 4-month lysozyme levels in Group 2 were the same as found at 9 months and 12 months of age in both Groups 1 and 2. Juvenile channel catfish produce lysozyme at 2 months equivalent to the lysozyme concentrations produced at 9 months and 12 months in both Groups 1 and 2. Total protein and albumin concentrations were determined for both Groups 1 and 2. Albumin/Globulin (A/G) ratios were calculated by dividing albumin concentrations with globulin concentrations. A mean of 0.7 for the A/G ratios was found for 4, 6, 9, and 12 month-old channel catfish. This indicates a satisfactory level for A/G ratio in channel catfish. It was found that juvenile channel catfish are competent in producing mean concentrations of the innate immune proteins MBL and lysozyme at 2 months of age that were comparable with the concentrations found for 12 month-old channel catfish and this was statistically significant ($p < 0.05$). The greatest increase in mean MBL and mean lysozyme was seen at 4 months of age in the month of September. The lowest levels of MBL (10.6 μg/ml) were seen in the months of November and February, and the highest level of MBL (26.9 μg/ml) was seen in September. The highest total protein concentration (34.8 mg/ml) was seen at 12 months of age. A mean protein of 26.7 mg/ml was found for the 4, 6, 9, and 12 month-old catfish. This study provided a foundation and basis for understanding these innate immune proteins in the early immune response of channel catfish.

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Appendix

Table 1: Background corrected inverse density units and MBL (μg/ml) concentration levels in channel catfish aged 2, 4, 6, 9 and 12 months-old in Groups 1 and 2 that were one-year apart in their bleedings. Background corrected inverse density units were determined by Image-J analysis by measuring the density dots obtained by dot-blot enzyme linked immunosorbent assay. Quantitative analysis of MBL concentrations were obtained from a standard curve of background corrected inverse density units vs. MBL concentrations (μg/ml). Number in parenthesis is the Group 1 or 2 year in which the channel catfish were bled.

Channel Catfish Age in Months Groups 1 and 2	**Background Corrected Inverse Density Units**	**Mannose-Binding Lectin (μg/ml)**
2 (1)	35.7	19.6
2 (2)	39.3	22.3
4 (1)	46.2	26.1
4 (2)	49.1	27.7
6 (1)	26.6	13.1
6 (2)	19.6	8.0
9 (1)	20.8	8.9
9 (2)	25.4	12.2
12 (1)	36.5	20.2
12 (2)	35.5	19.5

Table 2: Mean background corrected inverse density units and mean MBL (μg/ml) concentration levels in channel catfish aged 2, 4, 6, 9 and 12 months-old in Groups 1 and 2 that were one-year apart in their bleedings. Means were calculated using the data in Table 1.

Channel Catfish Age in Months	**Mean Background Corrected Inverse Density Units Groups 1 and 2**	**Mean Mannose-Binding Lectin (μg/ml)**
2	37.5	21.0
4	47.7	26.9
6	23.1	10.6
9	23.1	10.6
12	36.0	19.9

Table 3. Standard curve data for lysozyme standards. Lysozyme standards used were prepared in 0.1 M sodium phosphate buffer, pH 7.2 using different concentrations of hen egg white lysozyme (Sigma, St. Louis, MO). Absorbance was obtained by UV-visible spectrometry measured at 450 nm.

Lysozyme Concentration (μg/ml)	**Absorbance 450 nm**
100	0.0025
50	0.0012
25	0.0007
12.5	0.0004
6.25	0.0003
3.13	0.0002
1.56	0.0002
0.78	0.0001
0.39	0.0001

Table 4: Lysozyme concentrations (μg/ml) of Groups 1 and 2 of channel catfish aged 2, 4, 6, 9 and 12 months-old that were one-year apart in their bleedings. Concentrations were measured by lysozyme turbidometry assay. Number in parenthesis is the Group 1 or 2 year in which the channel catfish were bled.

Channel Catfish Age in Months Groups 1 and 2	**Lysozyme Concentration (μg/ml)**
2 (1)	10
2 (2)	5
4 (1)	20
4 (2)	5
6 (1)	15
6 (2)	10
9 (1)	5
9 (2)	5
12 (1)	5
12 (2)	5

Table 5. Mean lysozyme concentrations (μg/ml) of Groups 1 and 2 of channel catfish aged 2, 4, 6, 9 and 12 months-old that were one-year apart in their bleedings. Means were calculated from the data in Table 4.

Channel Catfish Age in Months	**Mean Lysozyme Concentrations Groups 1 and 2 (μg/ml)**
2	7.5
4	12.5
6	12.5
9	5
12	5

Table 6: Total protein concentrations (mg/ml), albumin concentrations (mg/ml), globulin concentrations (mg/ml) and albumin/globulin ratios of Groups 1 and 2 channel catfish aged 2, 4, 6, 9 and 12 months-old that were one-year apart in their bleedings. Total protein concentrations (mg/ml) were done using the room temperature protocol bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL). Albumin concentrations (mg/ml) were calculated based on the protocol of Pointe Scientific assay (Fisher Scientific, Pittsburg, PA). Globulin concentrations (mg/ml) were calculated by subtracting the albumin concentrations from the total protein concentrations. Albumin/globulin ratios were calculated by dividing albumin concentrations (mg/ml) with the globulin concentrations (mg/ml). Number in parenthesis is the Group 1 or 2 year in which the channel catfish were bled. ND, not done, insufficient serum

Channel Catfish Age in Months Groups 1 and 2	**Total Protein Concentrations (mg/ml)**	**Albumin Concentrations (mg/ml)**	**Globulin Concentrations (mg/ml)**	**Albumin/Globulin (A/G) Ratios**
2 (1)	ND	ND	ND	ND
2 (2)	ND	ND	ND	ND
4 (1)	27.2	11.7	15.5	0.75
4 (2)	27.6	11.0	16.6	0.66
6 (1)	23.0	7.9	15.1	0.52
6 (2)	20.0	9.7	10.3	0.94
9 (1)	24.3	10.9	13.4	0.81
9 (2)	22.0	8.5	13.5	0.63
12 (1)	34.3	10.4	23.9	0.44
12 (2)	35.3	17.4	17.9	0.97

Table 7: Mean total protein concentrations (mg/ml), albumin concentrations (mg/ml), globulin concentrations (mg/ml), albumin/globulin ratios of channel catfish aged 2, 4, 6, 9 and 12 months-old of Groups 1 and 2 that were one-year apart in their bleedings. Means were calculated using the data from Table 6. ND, not done, insufficient serum

Channel Catfish Age in Months	**Mean Total Protein Concentration Groups 1 and 2 (mg/ml)**	**Mean Albumin Concentration Groups 1 and 2 (mg/ml)**	**Mean Globulin Concentration Groups 1 and 2 (mg/ml)**	**Mean Albumin/Globulin (A/G) Ratio Groups 1 and 2**
2	ND	ND	ND	ND
4	27.4	11.4	16.1	0.71
6	21.5	8.8	12.7	0.69
9	23.2	9.7	13.5	0.72
12	34.8	13.9	20.9	0.67

Table 8. Background corrected inverse density units and mannose-binding lectin (μg/ml) concentration levels in channel catfish 5, 6 and 7 months-old. The background corrected inverse density units and MBL concentration levels at 5 and 6 months of age in Table 8 were similar to means of 4 and 6 month-old catfish (Table 2). The 7 month-old catfish data here were similar to the mean of 9 month-old catfish (Table 2). A serum pool of 10 channel catfish was used for each month.

Channel Catfish Age in Months	**Background Corrected Inverse Density Units**	**Mannose-Binding Lectin (μg/ml)**
5	46.3	26.2
6	20.3	8.7
7	26.7	13.2

Table 9. Mean monthly temperature variations of the innate immune proteins in two groups of channel catfish. Temperature Range 26.3 ^{O}C - 27.3 ^{O}C. Mean Temperature 26.8 ^{O}C.

Channel Catfish Age in months	**Month**	**Temperature (Degree Celsius)**	**Mean Mannose-Binding Lectin Concentrations (μg/ml)**	**Mean Lysozyme Concentrations (μg/ml)**
2	July	27.3	21.0	7.5
4	September	27.3	26.9	12.5
6	November	26.4	10.6	12.5
9	February	26.3	10.6	5
12	May	26.8	19.9	5

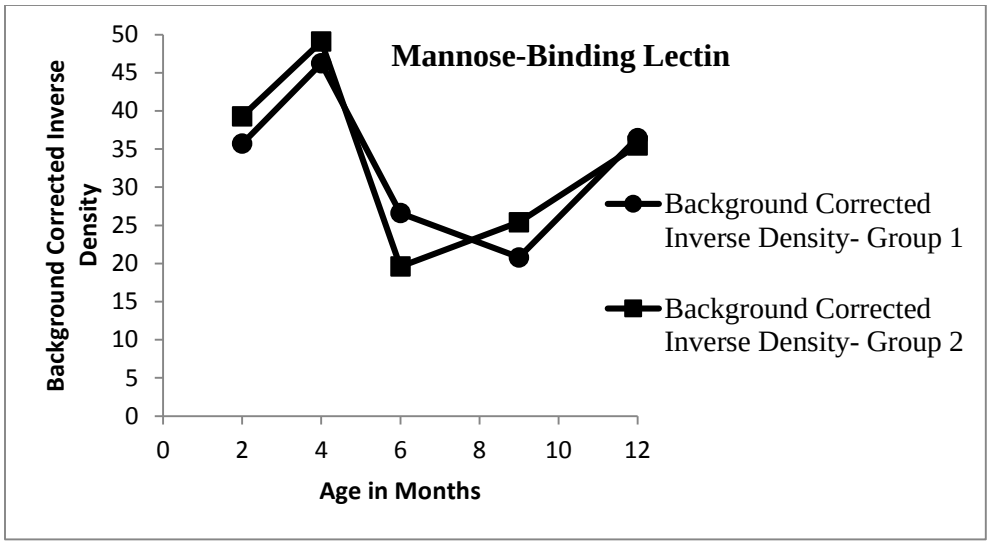

Figure 1. Background corrected inverse density units in Groups 1 and 2 in channel catfish aged 2, 4, 6, 9 and 12 months-old that were one-year apart in their bleedings as determined by Image-J analysis. An increase in background corrected inverse density units was seen at 4 months of age in both Groups 1 and 2. The graph was plotted in reference to the data in Table 1.

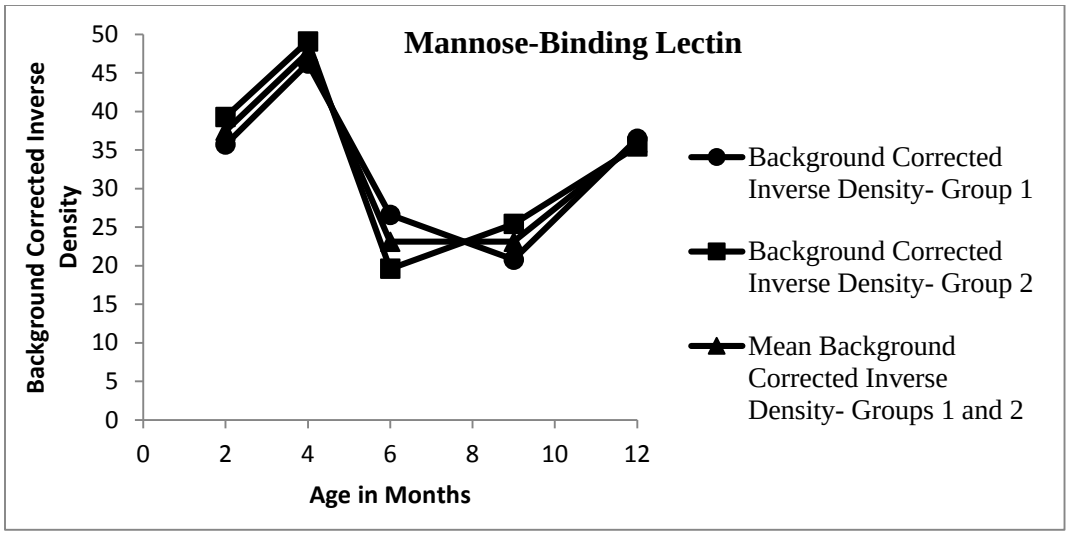

Figure 2. Background corrected inverse density units in Group 1, Group 2 and the mean background corrected inverse density units of Groups 1 and 2 in channel catfish aged 2, 4, 6, 9 and 12 months-old that were one-year apart in their bleedings as determined by Image-J analysis. An increase in mean background corrected inverse density units was seen at 4 months of age. Mean background corrected inverse density units were similar in 6 and 9 months-old catfish. The 2 month and 12 month-old catfish were nearly similar in their mean background corrected inverse density units. The graph was plotted in reference to the data in Tables 1 and 2.

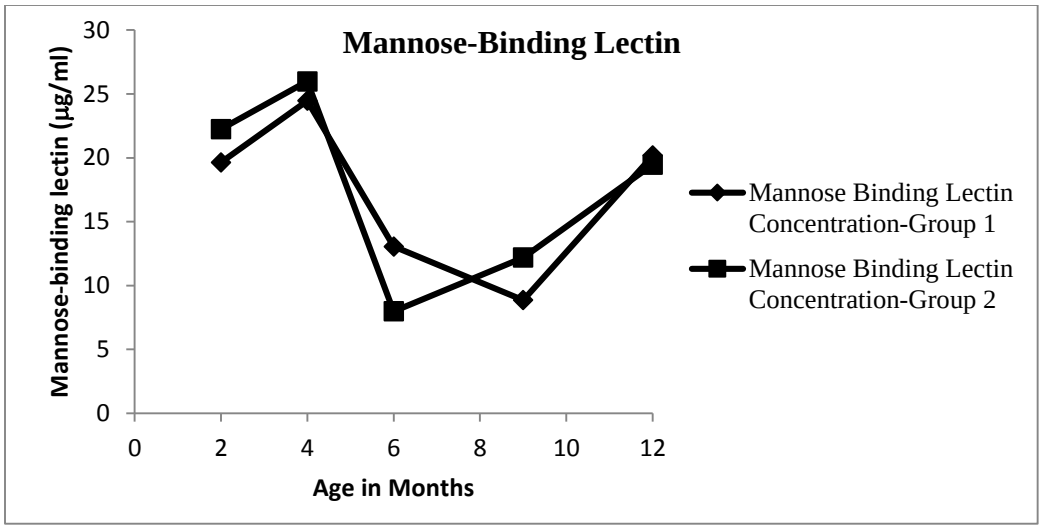

Figure 3. Mannose-binding lectin concentrations (µg/ml) were determined in channel catfish aged 2, 4, 6, 9 and 12 months-old of Groups 1 and 2 that were one-year apart in their bleedings. A standard curve for background corrected inverse density units vs. MBL concentrations (µg/ml) was used to quantitatively determine the MBL concentrations. An increase in MBL concentrations (µg/ml) was seen at 4 months of age in both Groups 1 and 2. Mannose-binding lectin levels at 2 months of age were very comparable with catfish at 12 months of age. The graph was plotted in reference to the data in Table 1.

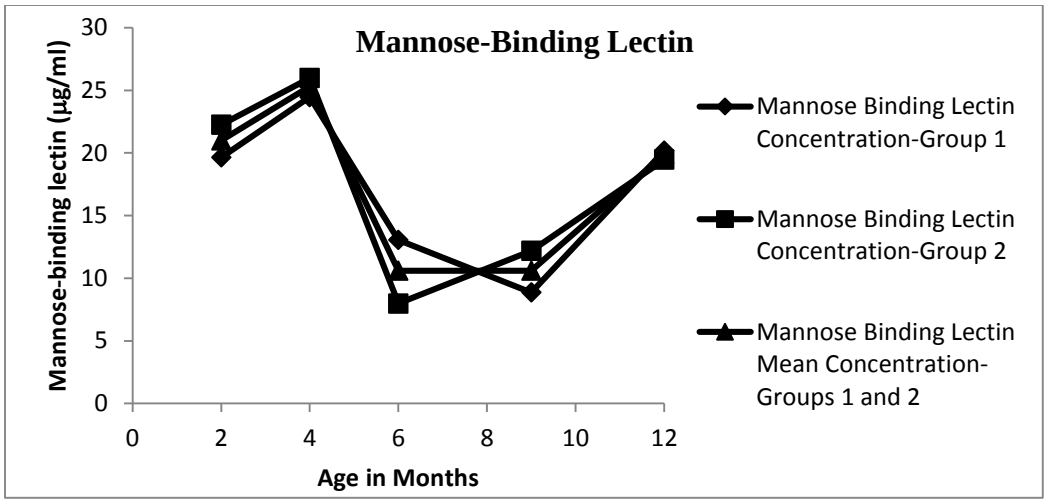

Figure 4: Mannose-binding lectin concentration data (µg/ml) in channel catfish aged 2, 4, 6, 9 and 12 months-old of Group 1, Group 2 and mean MBL concentrations of both Groups 1 and 2 that were one-year apart in their bleedings. A standard curve for background corrected inverse density units vs. MBL concentrations (µg/ml) was used to quantitatively determine MBL concentrations. An increase in mean MBL concentration (µg/ml) was seen at 4 months of age. Mean MBL concentrations (µg/ml) were similar in 6 and 9 months-old catfish. The graph was plotted in reference to the MBL concentration data in Tables 1 and 2.

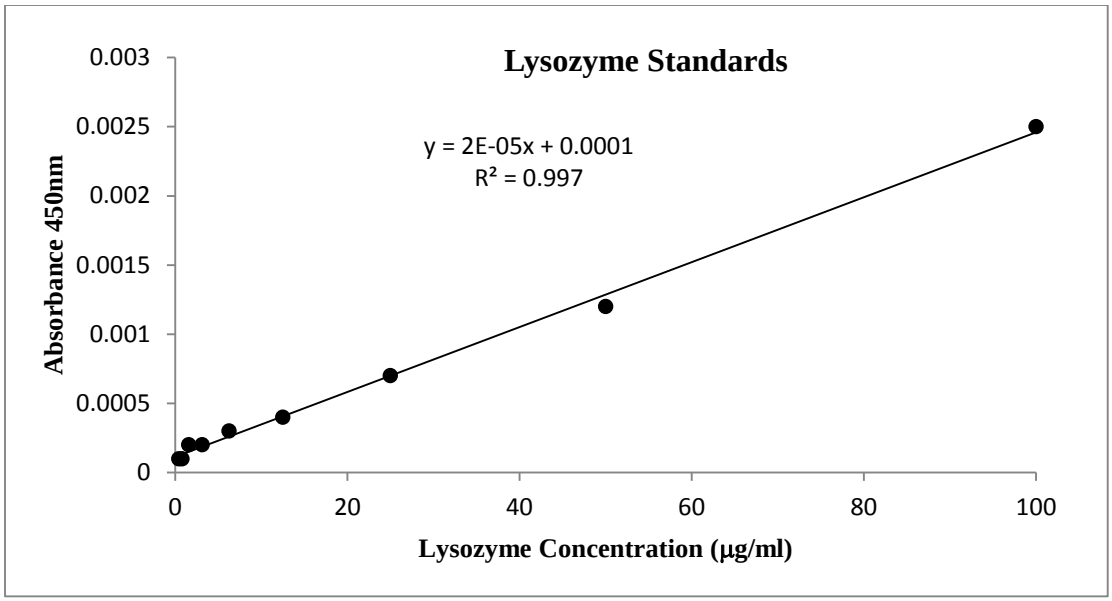

Figure 5. A standard curve was plotted between different concentrations of lysozyme standards (µg/ml) vs. absorbance at 450 nm. Absorbance of the lysozyme standards (hen egg white lysozyme) was measured at 450 nm. The R^2 value of the standard curve obtained was 0.997 and the slope was y = 2E-05x + 0.0001. The graph was plotted in reference to the data in Table 3.

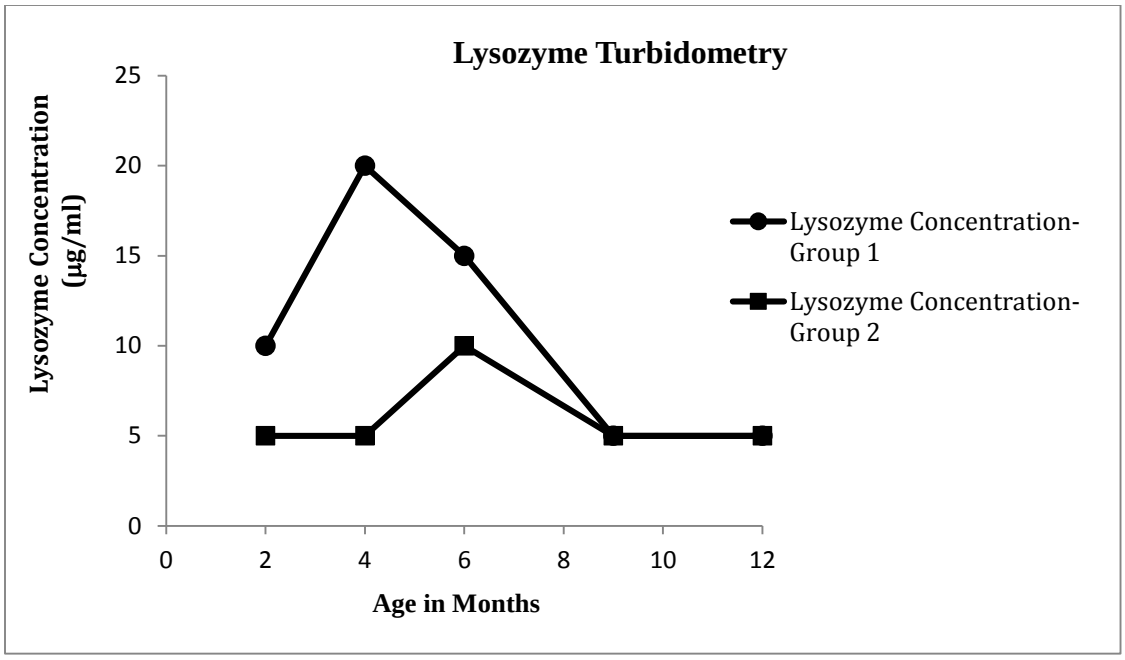

Figure 6. Lysozyme concentrations (µg/ml) in Groups 1 and 2 of channel catfish aged 2, 4, 6, 9 and 12 months-old that were one-year apart in their bleedings. Absorbances of the different ages were obtained by turbidometry assay and the concentrations were calculated from the slope of the standard lysozyme curve (Fig. 5). Group 1 showed higher variations in lysozyme concentrations when compared with Group 2. The highest rise in lysozyme concentration was seen in Group 1 with 4 month-old catfish. A rise in lysozyme concentrations was seen at 6 months of age in both Groups 1 and 2. The graph was plotted in reference to the data in Table 4.

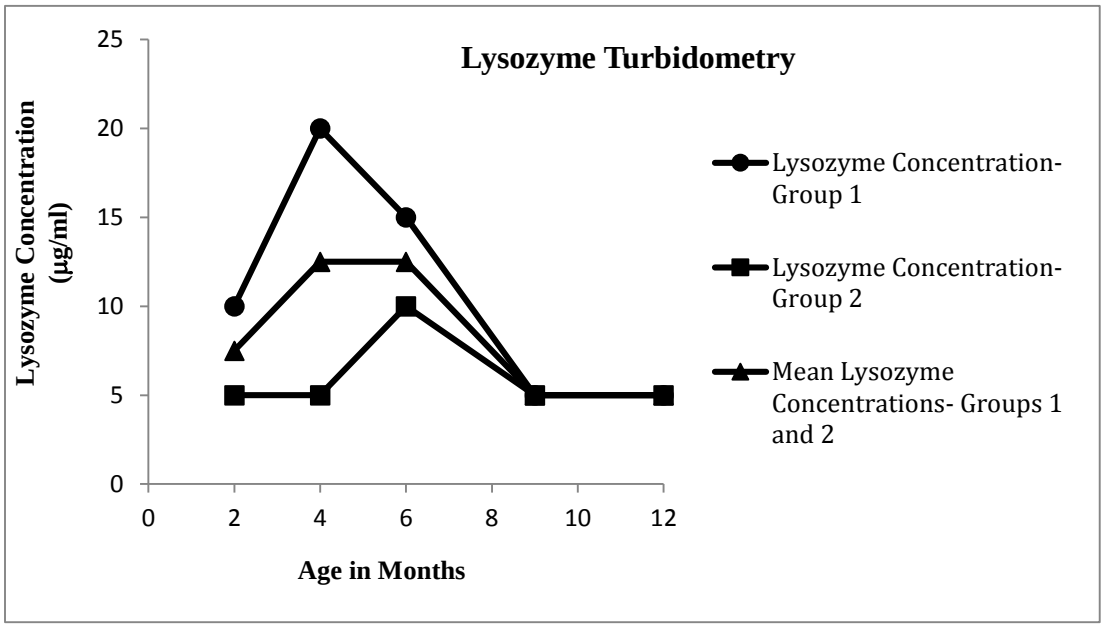

Figure 7: Lysozyme concentrations (µg/ml) in channel catfish aged 2, 4, 6, 9 and 12 months-old of Groups 1 and 2 and mean lysozyme concentrations of both Groups 1 and 2 that were one-year apart in their bleedings. Absorbances of the different ages were obtained by turbidometry assay and concentrations were calculated from the slope of a standard lysozyme curve. The highest rise in mean lysozyme concentrations was seen in 4 and 6 month-old catfish. Lysozyme levels at 2 months of age were comparable with those at 12 months of age. The mean lysozyme concentrations were the same in 9 and 12 month-old catfish. The graph was plotted in reference to the data in Tables 4 and 5.